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# A liquid chromatographic method for the determination of danazol in human serum

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**Abstract**: A liquid chromatographic method for the determination of danazol in human serum has been developed. Reversed-phase $C_8$ and $C_{18}$ columns were used with a column-switching valve, isocratic elution and UV detection. Sample pretreatment involved extraction of the drug with pentane–methylene chloride. The method enabled the measurement of the drug at a concentration as low as 1 ng $ml^{-1}$, with precision of 15.0% and accuracy of 8.3%. The method was used to run a two way replicated pharmacokinetic study of danazol. The main pharmacokinetic parameters were (mean of two periods): $AUC_{inf}$ = 480.94 ng × h $ml^{-1}$, $C_{max}$ = 53.2 ng $ml^{-1}$, $t_{max}$ = 2.5 h, $t_{0.5}$ = 18.00 h.

**Keywords**: *Danazol; reversed-phase chromatography; column switching; pharmacokinetics; human serum.*

## Introduction

Danazol, a steroidal isoxazol, inhibits pituitary output of gonadotrophins and suppresses the pituitary–ovarian axis. It is used in the treatment of benign breast disorders and endometreosis in doses of 100–800 mg daily. Danazol has several metabolites which do not exhibit pharmacological activity of the parent drug [1]. Pharmacology and pharmacokinetics of the drug have been reviewed by Potts *et al.* [2].

Two main approaches for the quantification of danazol in human serum or plasma have been employed previously: radioimmunoassay (RIA) [3–6] and liquid chromatography (LC) [7–9]. The most sensitive RIA method is the one published by Peterson *et al.* [3], which has a limit of detection of 2 ng $ml^{-1}$. Cross-reactivity with structurally similar compounds may sometimes cause lack of specificity of RIA methods. In 1987, two very similar LC methods for danazol in serum and plasma were published [7, 8]. Both employed liquid extraction with hexane, reversed-phase chromatography, UV detection at 285–287 nm and each reported limits of detection of 1.5–2 ng $ml^{-1}$. An earlier LC method had a limit of determination of 10 ng $ml^{-1}$ [9].

One of the remaining unresolved issues relating to the danazol pharmacokinetics is its half-life in man, which, according to respective papers, was 29 h [2], 4.5 h [4], 5.97 h [8], and 14.7 h [3]. All researchers reported substantial inter-subject variability in the pharmacokinetic profiles.

There were two objectives in this study. The first objective was to develop a quick and sensitive danazol assay in human serum, which could be used for pharmacokinetic and relative bioavailability purposes. The second objective was to evaluate inter- and intra-subject variability of danazol pharmacokinetics after administration of a single oral dose.

## Experimental

### *Reagents*

Danazol and 1,4-androstadiene-3-17-dione, used as the internal standard, were purchased from Sigma (St. Louis, MO, USA). All other chemicals were HPLC grade and were used without further purification.

### *Apparatus*

The chromatographic system consisted of a Waters Model 590 programmable solvent delivery module, a solvent delivery system

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6000 A, a Waters WISP 710B autosampler, Model 481 UV detector and automated switching valve; all from Waters (Milford, MA, USA).

Two columns per system were used. Both columns were packed in this laboratory, the first with Spherisorb C8 (5 μm, 150 × 3.9 mm i.d.) and the second with ODS-2 Spherisorb, (5 μm, 150 × 4.6 mm). Both packing materials were obtained from PhaseSep (Norfolk, CT, USA). Chromatograms were recorded using a SE-120 strip chart recorder (BBC Goerz Metrawatt (Mississauga, Ontario, Canada). Data were collected and processed on a 3357 Laboratory Automation system (Hewlett Packard, Avondale, PA, USA).

*Chromatographic conditions*

A schematic of the chromatographic system is shown in Fig. 1. The mobile phase was prepared by mixing 1800 ml of water with 2200 ml of acetonitrile. After stirring, the mobile phase was filtered through a 0.45 μm nylon filter. The flow rate was 1.6 ml $min^{-1}$ for both pumps; the same mobile phase was pumped by both pumps. The detector was set at 285 nm, 0.005 AUFS. Retention times were 9.5 and 4.2 min for the danazol and the internal standard, respectively. The total run time was 14 min. The whole system was maintained at a room temperature of 22 ± 3°C.

*Standard and quality control preparation*

The standards and quality control samples (QCs) were prepared by adding appropriate volumes of methanolic spiking solution of danazol to human serum. The volume added was always smaller than or equal to 2% of total volume of the sample, so that the integrity of the serum was maintained. The QCs were prepared in the same way, using separately weighed stock solutions. After aliquoting, 1 ml samples were stored at −15°C until required.

*Clinical pharmacokinetic study*

Six healthy, sterile, pre-menopausal female volunteers, 18 to 45 years of age, weighing at least 50 kg and not receiving any medication for the 7 days preceding the study participated in the project after giving a written informed consent. A single dose of 200 mg of danazol (Cyclomen®, Winthrop, USA) was administered orally with 250 ml of water, and blood samples were collected at 0.0, 2, 3, 4, 5, 8, 12, 24, 36, 48, 60, 72 and 84 h. The same volunteers took part in the second part of the project after 14 days of drug wash-out. The second period was conducted in exactly the same way as the first.

*Method of extraction*

Internal standard was added (100 μl of 1,4-androstadiene-3,17-dione, 10 μg $ml^{-1}$) to 1.0 ml of standard, QCs or clinical sample. After adding 5 ml of pentane–methylene chloride (4:1, v/v), the tubes were shaken for 10 min on a reciprocal shaker at 150 rpm and then centrifuged at room temperature for 10

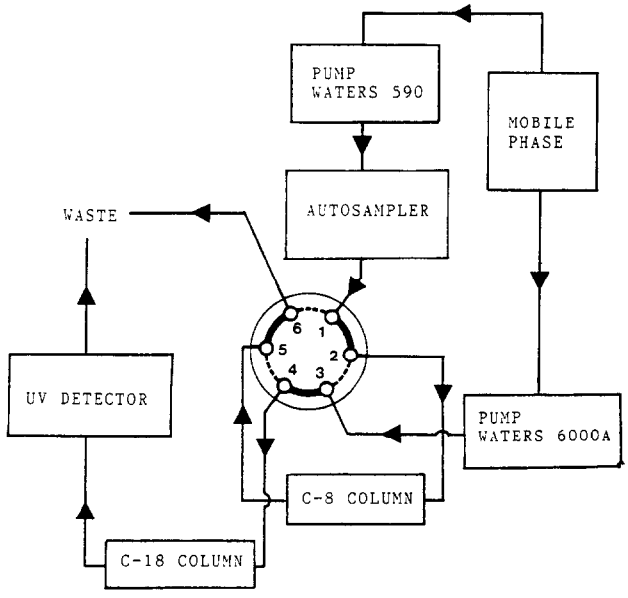


**Figure 1**
Chromatographic system.

min at 1000*g*. The upper organic layer was transferred into a new tube and evaporated under a stream of nitrogen. The residue was dissolved in 200 μl of mobile phase.

## Results and Discussion

*Development of a column-switching system*

A column-switching technique was used in this assay, to achieve better resolution of the drug and endogenous materials and the elimination of late-eluting peaks. Figure 2 shows a chromatogram of 100 ng $ml^{-1}$ danazol in a serum extract after separation on the $C_8$ column alone. Better resolution of the peaks in close proximity to danazol was needed. This could be achieved either by using a $C_{18}$-column or by reducing the concentration of organic modifier in the mobile phase. However, the large, unidentified, endogenous peak at about 15.5 min would have been eluted at about 40 min, drastically decreasing the output of the system. The problem of late-eluting peaks was clearly encountered as well by Nygard *et al.* [7] and solved by linear gradient elution.

A column switching procedure was used to solve this problem. At the time marked in Fig. 2 by "a" the eluent from column $C_8$ was redirected to the $C_{18}$ column, until time indicated by "b". In this way, the $C_{18}$ column separated the danazol peak from adjacent peaks, while at the same time, the $C_8$ column was rinsed and prepared for the next injection.

Chromatograms obtained while using the described procedure are shown in Fig. 3. Panel A shows a drug-free serum, panel B shows a subject plasma, 4.0 h after drug administration and panel C represents a plasma spiked at a concentration of 10.0 ng $ml^{-1}$.

*Precision and accuracy*

A set of seven calibration standards, a set of three QCs in duplicate, a zero-time sample and a blank were analysed with every batch of clinical samples. The within-run and between-run precision and accuracy of the assay are shown in Table 1. The danazol to internal standard peak height ratio was linearly related to concentration over the range 1.0–150 ng $ml^{-1}$. A linear regression analysis using a least-

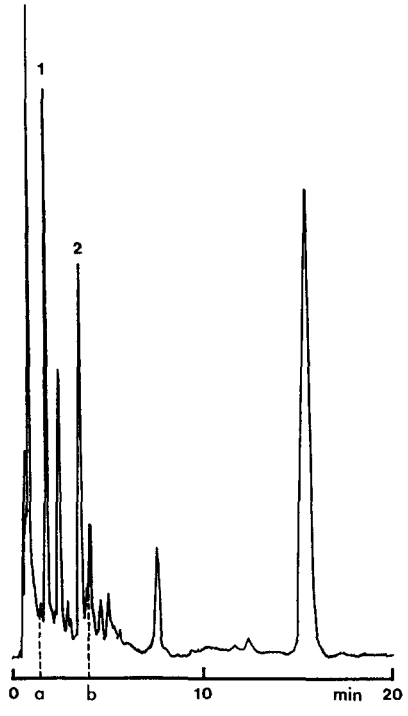


**Figure 2**
Chromatography of extracted danazol (100 ng $ml^{-1}$) after $C_8$ column only. Portion of the eluent between points "a" and "b" was sent to $C_{18}$ column, where the danazol peak was well resolved from other peaks. In the meantime, the endogenous peak at 15.5 min was eluted from the $C_8$ column and next sample is injected.

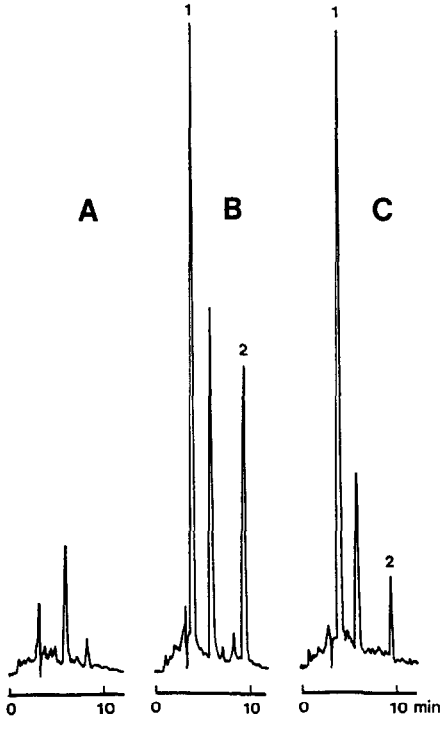


**Figure 3**
Representative chromatograms of the danazol in serum (two-column system) (A) drug-free serum, (B) serum obtained 4 h after 200 mg danazol administration, (C) standard 10 ng $ml^{-1}$. Chromatographic conditions: wavelength, 285 nm, 0.005 AUFS; mobile phase, water–acetonitrile (45:55, v/v); flow rate, 1.6 ml $min^{-1}$ 1, internal standard; 2, danazol.

**Table 1**
Precision and accuracy of the danazol serum assay

| Sample concentration (ng ml$^{-1}$) | $n$ | Mean | ±SD | RSD (%) | Accuracy* |
|---|---|---|---|---|---|
| Within-run | | | | | |
| 1.00 | 9 | 1.083 | 0.1620 | 15.0 | 108.3 |
| 3.00 | 10 | 2.096 | 0.3134 | 10.8 | 96.7 |
| 60.0 | 9 | 59.01 | 3.626 | 6.1 | 98.4 |
| 135 | 9 | 135.7 | 5.45 | 4.0 | 100.5 |
| Between-run | | | | | |
| 3.00 | 7 | 2.924 | 0.3239 | 1.1 | 97.5 |
| 60.0 | 7 | 57.05 | 2.081 | 3.6 | 95.1 |
| 135 | 7 | 131.7 | 4.31 | 3.3 | 97.6 |

*Calculated from found concentration/nominal concentration × 100.

squares fit was performed with the reciprocal of the drug concentration as a weighting factor. The correlation coefficients, an indication of goodness of fit, were equal to or better than 0.9977 for six curves. The RSD was always less than 15.0% and the deviation from nominal concentration, a measure of accuracy, was not greater than 8.3% at the lower limit of quantification. At 135 ng ml$^{-1}$, the RSD was always less than 4.4% and the deviation from the nominal value was 1.2%. The signal to noise ratio at a concentration of 1.0 ng ml$^{-1}$ was 3.

*Recovery*

Recovery was calculated by comparing extracted QCs with a separately prepared calibration curve. The recovery of danazol at 3.0 ng ml$^{-1}$ was 99.5% (RSD = 6.7%, $n$ = 5), and at 90 ng ml$^{-1}$ was 107.7% (RSD = 2.9%, $n$ = 6). Recovery of the internal standard was 100.0% (RSD = 4.5%, $n$ = 6).

*Pharmacokinetics*

The following parameters were determined: Area under the curve (AUC), area under the curve extrapolated ($AUC_{inf}$), peak concentration ($C_{max}$), time to the peak ($t_{max}$), elimination rate constant ($K_{el}$) and elimination half-life ($t_{0.5}$) [10].

The trapezoidal rule was used to calculate the AUC until the final detectable plasma concentration. The residual area, extrapolated to infinity, was calculated by the dividing of the final concentration by $K_{el}$ and then added to the AUC. The apparent elimination half-life was calculated directly from $K_{el}$. The results are summarized in Table 2, while the mean plasma profiles are shown in Fig. 4.

Intra-subject variation of $AUC_{inf}$, $C_{max}$, $t_{max}$ and $t_{0.5}$ was 243, 208, 200 and 338%, respectively. Inter-subject variation was greater and reached 456, 474, 200 and 400%, respectively. In contrast, the study showed that there was no statistically significant difference in the mean pharmacokinetic parameters determined in the first period compared with the second.

The pharmacokinetic profiles shown in Fig. 4 clearly demonstrate biphasic elimination of danazol and help to explain the large variation in the reported half-lives of danazol. Should the danazol concentration be monitored for less than 12 h after dosing, then only the first elimination phase of the drug will be detected, with a half-life of 4–5 h (3.4 and 3.9 h in this study).

If the monitoring is continued for an additional 2–3 days, the terminal phase of elimination would be demonstrated with a half-life ranging from 15 to 30 h. Such a biphasic pharmacokinetic profile is shown in the paper of Nygard *et al.* [7] and marked in papers of Hooper [8] and Peterson [3]. The profile presented by Potts [2] did not show the second elimination phase, yet the half-life of 29 h was reported.

Generally, whereas the $C_{max}$ values provided by RIA and LC seem to be in good agreement, the danazol concentrations measured by RIA are higher at later sampling times (>10 h) with those values obtained by LC. For example the RIA method gave values of 38 ng ml$^{-1}$ [2] (200 mg dose) and 25 ng ml [4] (200 mg dose) after 24 h, but LC gave values of 7 ng ml$^{-1}$ [8] (400 mg dose) and 3 ng ml$^{-1}$ [7] (400 mg dose). The LC publication of Yasuda *et al.* [9] was less helpful for such a comparison because its limit of determination was 10 ng ml$^{-1}$ and the last sampling time was

**Table 2**
Pharmacokinetic parameters for danazol in human serum after a 200 mg dose

| Subject | Period | AUC (h ng ml$^{-1}$) | $AUC_{inf}$ (h ng ml$^{-1}$) | $C_{max}$ (ng ml$^{-1}$) | $t_{max}$ (h) | $K_{el}$ | $t_{0.5}$* (h) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 211.79 | 231.97 | 36.5 | 2 | 0.063 | 11.0 |
| | 2 | 503.73 | 562.75 | 76.0 | 2 | 0.022 | 31.5 |
| 2 | 1 | 530.19 | 573.35 | 53.3 | 2 | 0.025 | 27.7 |
| | 2 | 600.23 | 658.80 | 58.8 | 2 | 0.022 | 31.5 |
| 3 | 1 | 385.66 | 434.27 | 55.2 | 2 | 0.026 | 26.7 |
| | 2 | 355.36 | 371.26 | 58.0 | 3 | 0.087 | 8.0 |
| 4 | 1 | 784.25 | 831.25 | 99.6 | 2 | 0.028 | 24.8 |
| | 2 | 783.16 | 853.57 | 82.2 | 2 | 0.024 | 28.9 |
| 5 | 1 | 415.73 | 461.90 | 43.4 | 2 | 0.022 | 31.5 |
| | 2 | 322.21 | 339.16 | 26.8 | 4 | 0.044 | 15.8 |
| 6 | 1 | 171.68 | 187.27 | 21.0 | 4 | 0.051 | 13.6 |
| | 2 | 247.08 | 265.63 | 27.8 | 3 | 0.041 | 16.9 |
| Mean (±SD) | 1 | 416.55 (224.04) | 453.34 (235.57) | 51.50 (26.65) | 2.3 (0.82) | 0.036 (0.017) | 19.3 |
| | 2 | 468.63 (200.42) | 508.53 (224.10) | 54.93 (23.41) | 2.7 (0.82) | 0.040 (0.025) | 17.3 |

*Mean $t_{0.5}$ calculated directly from $0.693/k_{el}$.

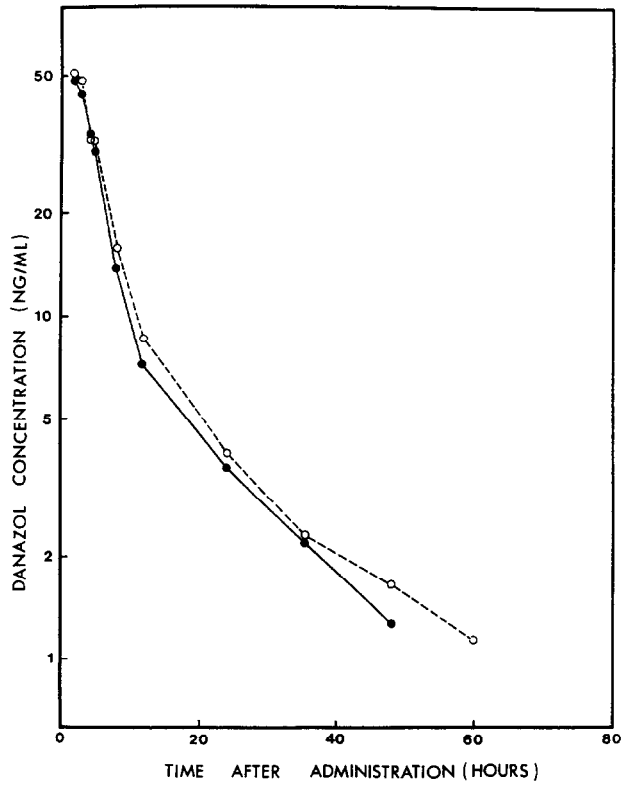


**Figure 4**
Concentration–time profiles for danazol in serum after a 200 mg dose. —●—, First period; —○— second period.

12 h. In the present study, 3.4–4.0 ng $ml^{-1}$ danazol was found 24 h after a 200 mg dose. Although the RIA methods were reportedly checked for cross-reactivity with related compounds, the differences in the values obtained by LC and RIA are a cause for concern and cross-reactivity with a compound not considered previously is a possible explanation.

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